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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Neurogenic differentiation by hippocampal neural stem and progenitor cells is biased by NFIX expression

Lachlan Harris[1,*], Oressia Zalucki[1,*], Olivier Clément[2], James Fraser[1], Elise Matuzelski[1], Sabrina Oishi[1], Tracey J. Harvey[1], Thomas H. J. Burne[3,4], Julian Ik-Tsen Heng[2], Richard M. Gronostajski[5] and Michael Piper[1,3,‡]

ABSTRACT

Our understanding of the transcriptional programme underpinning adult hippocampal neurogenesis is incomplete. In mice, under basal conditions, adult hippocampal neural stem cells (AH-NSCs) generate neurons and astrocytes, but not oligodendrocytes. The factors limiting oligodendrocyte production, however, remain unclear. Here, we reveal that the transcription factor NFIX plays a key role in this process. NFIX is expressed by AH-NSCs, and its expression is sharply upregulated in adult hippocampal neuroblasts. Conditional ablation of *Nfix* from AH-NSCs, coupled with lineage tracing, transcriptomic sequencing and behavioural studies collectively reveal that NFIX is cell-autonomously required for neuroblast maturation and survival. Moreover, a small number of AH-NSCs also develop into oligodendrocytes following *Nfix* deletion. Remarkably, when *Nfix* is deleted specifically from intermediate progenitor cells and neuroblasts using a *Dcx-creER^{T2}* driver, these cells also display elevated signatures of oligodendrocyte gene expression. Together, these results demonstrate the central role played by NFIX in neuroblasts within the adult hippocampal stem cell neurogenic niche in promoting the maturation and survival of these cells, while concomitantly repressing oligodendrocyte gene expression signatures.

KEY WORDS: Adult neurogenesis, Hippocampus, Neural stem cell, Neuroblast, Oligodendrocyte, Mouse

INTRODUCTION

NFIX is expressed during mouse nervous system development and governs neural stem/progenitor cell fate (Harris et al., 2015; Fane et al., 2017). In the developing dorsal telencephalon (Campbell et al., 2008; Heng et al., 2014; Harris et al., 2016) and cerebellum (Piper et al., 2011; Fraser et al., 2017) of mice, NFIX is essential for the timely differentiation of both neurons and astrocytes. The importance of NFIX for mouse brain development appears to be conserved during human brain development, as patients with *NFIX* mutations present with one of two developmental disorders characterised by a substantial brain phenotype: Malan syndrome, caused by loss-of-function *NFIX* mutations, and Marshall–Smith syndrome, caused by presumptive dominant-negative *NFIX* mutations (Malan et al., 2010; Sotos, 2014; Deciphering Developmental Disorders Study, 2017).

In the adult mouse (Gonçalves et al., 2016) and human brain (Spalding et al., 2013), neural progenitor cells persist within the dentate gyrus of the hippocampus, where they generate dentate granule neurons that contribute to learning and memory, as well as mood regulation. We have shown previously that mice heterozygous for *Nfix* exhibit abnormal neurogenesis and functional deficits in a hippocampal-dependent learning and memory task (Harris et al., 2013). However, as NFIX has been shown to modulate the embryonic and postnatal development of the hippocampus, in part via the regulation of intermediate progenitor cell specification, it is not possible to determine the function of NFIX in adult neurogenesis using heterozygous mice. Given that NFIX is highly expressed in adult hippocampal progenitor cells (Harris et al., 2013; Shin et al., 2015; Gao et al., 2016; Chen et al., 2017), and that developmentally important proteins often execute similar differentiation programmes within adult hippocampal progenitor cells (Urban and Guillemot, 2014), we sought to address the hypothesis that NFIX is specifically required for neurogenesis in the adult hippocampus.

Here, we use inducible *cre*-recombinase drivers and lineage tracing to test this hypothesis. We reveal that, contrary to expectation, the removal of *Nfix* has only a limited effect on adult hippocampal neural stem cells (AH-NSCs) themselves. Importantly, however, we demonstrate that NFIX expression is cell-autonomously required for the maturation and survival of immature neurons (neuroblasts) generated by AH-NSCs. Deletion of *Nfix* from AH-NSCs (*nestin-creER^{T2}*) resulted in the generation of neuroblasts that fail to extend a dendritic branch and to mature into dentate granule neurons. Moreover, whereas wild-type AH-NSCs mostly generated neurons and occasionally astrocytes, we found that a small proportion of *Nfix*-deficient AH-NSCs also generated oligodendrocytes. Remarkably, these phenotypes were recapitulated when *Nfix* was conditionally ablated from the lineage-committed progeny of AH-NSCs (intermediate progenitor cells and neuroblasts; *Dcx-creER^{T2}*), with these *Nfix*-deficient cells displaying morphological defects and increased mRNA expression of oligodendrocyte precursor genes. These results demonstrate that NFIX is required for neuroblast maturation and survival within the adult hippocampus. We also reveal the novel finding that NFIX suppresses oligodendrocyte gene expression within cells that are considered to be neuronally committed within the dentate gyrus, namely intermediate progenitor cells and neuroblasts. Collectively, these data enhance our understanding of the gene regulatory networks governing neural stem and progenitor cell fate

[1]The School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland, Australia 4072. [2]Curtin Health Innovation Research Institute, Curtin University, Bentley, Western Australia, Australia 6102. [3]Queensland Brain Institute, The University of Queensland, Brisbane, Australia 4072. [4]Queensland Centre for Mental Health Research, The Park Centre for Mental Health, Wacol, Queensland, Australia 4076. [5]Department of Biochemistry, Program in Genetics, Genomics and Bioinformatics, Center of Excellence in Bioinformatics and Life Sciences, State University of New York at Buffalo, Buffalo, NY 14203, USA.
*These authors contributed equally to this work

‡Author for correspondence (m.piper@uq.edu.au)

M.P., 0000-0002-6759-2560

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within the adult hippocampus, and reveal a previously unrecognised capacity for the progeny of AH-NSCs to exhibit developmental competence for oligodendrocytic differentiation resulting from the loss of *Nfix*.

RESULTS

NFIX is upregulated during neuronal differentiation

The pattern of NFIX expression was first assessed to provide clues as to the function of this protein during neurogenesis. The neurogenic lineage in the adult hippocampus comprises four main cell types. These are the mostly quiescent AH-NSCs, the highly proliferative intermediate progenitors (IPs), neuroblasts (immature neurons) and dentate granule neurons (Gonçalves et al., 2016). We have previously shown that NFIX is expressed by all of these cell types in the dentate gyrus and that its expression is particularly high in DCX^+ neuroblasts (Harris et al., 2013; Chen et al., 2017). However, a recent single-cell RNA-sequencing (RNA-seq) study found that there was a strong positive correlation between *Nfix* expression levels and the transition from an AH-NSC to an IP cell identity (Shin et al., 2015), suggesting that NFIX levels might increase prior to the neuroblast stage. To map accurately the expression levels of NFIX during adult hippocampal neurogenesis, we examined its expression in association with cell type-specific markers of lineage progression (Harris et al., 2018). We defined AH-NSCs as $SOX2^+$ cells positioned within the subgranular zone (SGZ) that were negative for TBR2 (EOMES) (Hodge et al., 2008) (91.74% of these cells expressed NFIX), IPs as $TBR2^+$ cells (100% of these cells expressed NFIX) and neuroblasts as DCX^+ cells (100% of these cells expressed NFIX). Consistent with previous data (Shin et al., 2015), we found that NFIX expression intensity was relatively low in AH-NSCs but was upregulated in IPs (P=0.0042) (Fig. S1). This high level of NFIX expression was maintained in neuroblasts, including those with a relatively mature morphology. Finally, dentate granule neurons, which we defined as cells positioned in the granule cell layer of the dentate gyrus (100% of these cells expressed NFIX), exhibited reduced expression of NFIX levels compared with IPs and neuroblasts (P=0.011) (Fig. S1E-H). These data show that NFIX expression levels peak as hippocampal neural progenitors undergo neuronal differentiation (Fig. S1I).

Efficient, inducible deletion of *Nfix* in *Nfix*^{iNestin} mice

$Nfix^{-/-}$ mice exhibit postnatal lethality and suffer from severe developmental defects, which limit their utility in assessing the role of NFIX in adult neurogenesis (Campbell et al., 2008). To circumvent this problem, we generated an inducible, loss-of-function mouse line by crossing mice containing a floxed *Nfix* allele (Messina et al., 2010) to an inducible *nestin-creER*^{T2} deletion strain (Imayoshi et al., 2006), generating $Nfix^{iNestin}$ or $Nfix^{control}$ mice. Tamoxifen administration to adult $Nfix^{iNestin}$ mice activated cre-recombinase in nestin-expressing progenitor cells (AH-NSCs and IPs). In adult (8- to 10-week-old) mice, 5 days post injection (dpi), NFIX was detected in only 39.3% of AH-NSCs and 12.9% of IPCs, compared with 91.7% and 100%, respectively, in $Nfix^{control}$ animals (Fig. S2). Therefore, the $Nfix^{iNestin}$ mouse is an efficient deletion strain with which to interrogate the function of NFIX in AH-NSCs.

NFIX is not required for the long-term maintenance of AH-NSCs

AH-NSCs are mostly quiescent, an adaptive feature of adult stem cells that ensures their long-term survival by protecting against the metabolic stress caused by cellular division (Valcourt et al., 2012). In a recent study using an *in vitro* model of neural stem cell quiescence, NFIX was shown to be enriched in enhancer regions specific to the quiescent state (Martynoga et al., 2013). Furthermore, NFIX overexpression was sufficient to induce quiescence in normally proliferating cells. These data led us to hypothesise that *Nfix* deletion in $Nfix^{iNestin}$ mice could lead to the premature depletion of the AH-NSC pool due to a loss of quiescence. Surprisingly, however, we found that *Nfix* deletion had no effect on total AH-NSC number ($SOX2^+$; $TBR2^-$) at 14 dpi, 120 dpi or even 1 year post-injection (Fig. 1A-D). We verified this observation using an alternative definition of AH-NSCs ($SOX2^+$ cells that extend a $GFAP^+$ process at least two-thirds of the way into the granule cell layer) (Seri et al., 2001; Steiner et al., 2006). Based on this definition, we again saw no effect of *Nfix* deletion on the total number of AH-NSCs in $Nfix^{iNestin}$ mice relative to controls (Fig. S3). These data demonstrate that NFIX deletion has no long-term effect on the maintenance of AH-NSCs.

As the total number of AH-NSCs was not altered following the depletion of *Nfix* from AH-NSCs, these data suggest that NFIX might not play as prominent a role in modulating quiescence *in vivo* as it does *in vitro* (Martynoga et al., 2013). We addressed this by examining the relative proliferation/quiescence of AH-NSCs in $Nfix^{iNestin}$ and control animals. At 14 dpi, there was an increase in the number of proliferating AH-NSCs in $Nfix^{iNestin}$ mice ($Ki67^+$; $SOX2^+$; $TBR2^-$) (Fig. 1E), supporting the *in vitro* observation that NFIX mediates quiescence (Martynoga et al., 2013). However, the effect size was small, and it did not lead to a detectable increase in the number of IPs at 14 dpi (Fig. 1F). Crucially, the effect was also transient, as there was no difference in the relative proliferation of AH-NSCs between groups at 120 dpi or 1 year post-injection (Fig. 1E), possibly because of niche homeostatic mechanisms or owing to redundancy from other NFI family members. Therefore, NFIX is not essential to maintain AH-NSC quiescence *in vivo*. Interestingly, at 120 dpi there was a small increase in the number of IPs in the dentate gyrus of *Nfix*-deficient mice; this, together with the increased expression of NFIX in IPs and neuroblasts (Fig. S1), implies that NFIX might be important for later aspects of lineage progression.

Neuroblasts fail to mature in *Nfix*^{iNestin} mice

During development, NFIX promotes neuronal differentiation, and abnormal expression is associated with multiple neurodevelopmental disorders (Malan et al., 2010; Yoneda et al., 2012; Heng et al., 2014; Harris et al., 2016; Deciphering Developmental Disorders Study, 2017). The increased expression of NFIX in IPs and neuroblasts suggests that NFIX might play a central role in regulating neuronal differentiation in the adult hippocampus. To investigate this, we examined the expression of the neuroblast marker DCX. At 14 dpi, there were significantly fewer neuroblasts in the $Nfix^{iNestin}$ dentate gyrus (53,981±1264 cells/mm^3) in comparison with controls (70,068±2972 cells/mm^3; P=0.01). At 45 dpi (P=0.0007) and 120 dpi (P=0.0002), there were one-third the number of DCX^+ cells in the $Nfix^{iNestin}$ dentate gyrus compared with the dentate gyrus of $Nfix^{control}$ mice (Fig. 2A-C). A higher proportion of these DCX^+ cells in $Nfix^{iNestin}$ mice co-labelled with cleaved-caspase-3, although this was not significant (P=0.11) (Fig. 2E). Of the remaining neuroblasts, many had an aberrant morphology. Only 16.6% of neuroblasts in $Nfix^{iNestin}$ mice extended a primary dendrite into the granule cell layer of the dentate gyrus compared with 77.5% of control neuroblasts at 45 dpi (P<0.0001), with a similar effect observed at 120 dpi (P=0.029) (Fig. 2D). Furthermore, a greater proportion of *Nfix*-deficient

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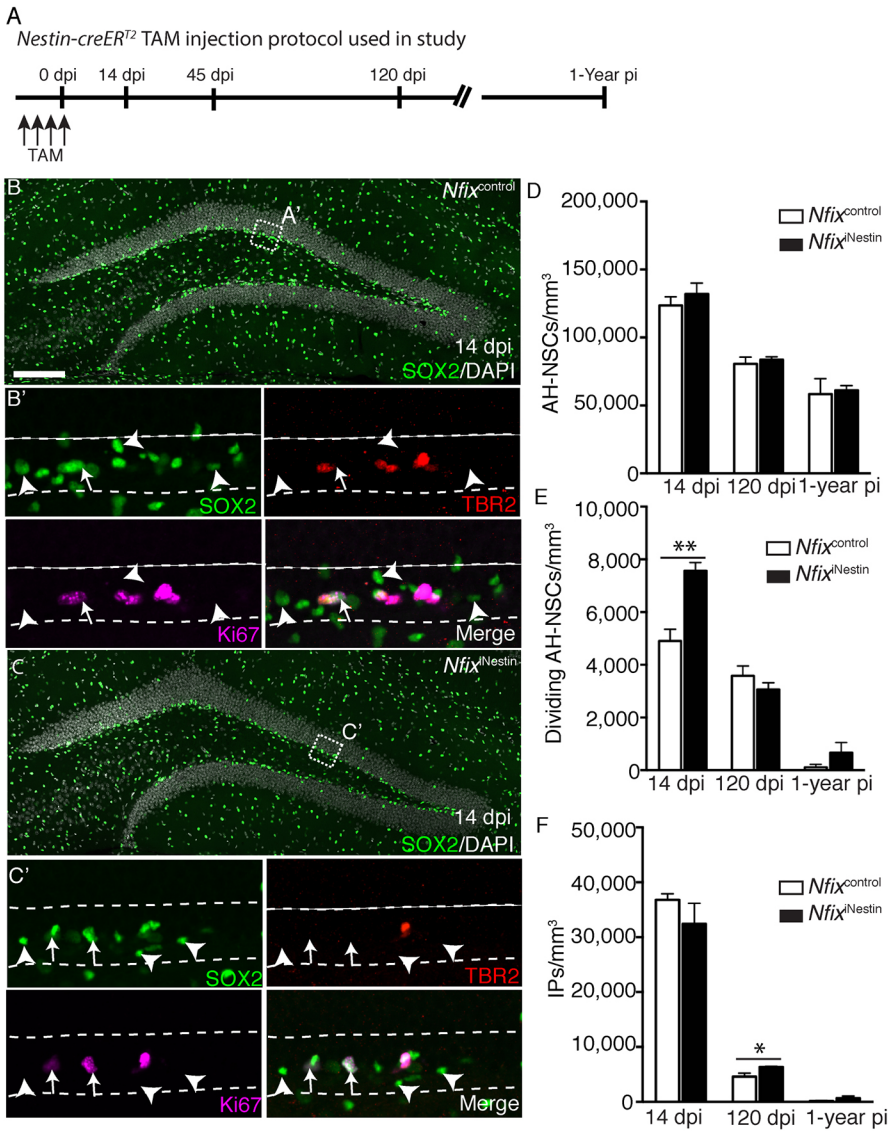

Fig. 1. NFIX deletion does not affect the long-term survival of AH-NSCs. (A) Schematic of the tamoxifen (TAM) regime used in *nestin-creER*^{T2} mice to delete *Nfix* from AH-NSCs. (B-C′) The dentate gyrus of *Nfix*^{control} (B,B′) and *Nfix*^{iNestin} (C,C′) mice at 14 dpi showing staining for DAPI (white) and SOX2 (green). Boxed regions in B and C are shown in panels B′ and C′, respectively. B′ and C′ show SOX2 (green), TBR2 (red) and Ki67 (magenta), with the dashed lines outlining the SGZ of the dentate gyrus. Marked cells in B′ and C′ indicate AH-NSCs that were SOX2^{+}; TBR2^{-}. Arrows indicate proliferating AH-NSCs (Ki67^{+}) and arrowheads indicate quiescent AH-NSCs (Ki67^{-}). (D) There was no effect of *Nfix* deletion on AH-NSC number at 14 dpi, 120 dpi or 1 year post-injection. (E) At 14 dpi there were more proliferating AH-NSCs in the *Nfix*^{iNestin} dentate gyrus than in controls, but this effect was not observed at 120 or 365 dpi. (F) IP number was unchanged at 14 dpi and was slightly increased at 120 dpi in *Nfix*^{iNestin} mice. *P<0.05, **P<0.01. Graphs depict mean±s.e.m. from six control and four *Nfix*^{iNestin} mice at 14 dpi, four mice per genotype at 120 dpi and three mice per genotype at 1 year post-injection. Scale bar (in B): 160 µm (B,C); 45 µm (B′,C′).

neuroblasts retained expression of the IP marker TBR2 at both 45 dpi (P=0.008) and 120 dpi (P=0.0011) (Fig. 2F). These data demonstrate that, in the absence of *Nfix*, neuroblasts fail to mature and that an increased proportion of them undergo programmed cell death.

Nfix^{iNestin} mice generate fewer mature granule neurons and have reduced performance in a hippocampal-dependent memory task

Over a period of 3-4 weeks, neuroblasts integrate into the existing hippocampal circuitry where they facilitate the formation of new memories (Gonçalves et al., 2016). Because surviving *Nfix*-deficient neuroblasts exhibit an aberrant and immature morphology, suggestive of impaired differentiation, we investigated whether these cells were capable of developing into mature dentate granule neurons. We injected bromodeoxyuridine (BrdU) daily (for 5 days) beginning 2 weeks after tamoxifen administration to label proliferating IPs, and sacrificed these animals 4 weeks after the final BrdU injection (Fig. 3A). In control animals, very few BrdU-labelled cells were DCX^{-}; NeuN (RBFOX3)$^{-}$ progenitors or DCX^{+} neuroblasts. Rather, the majority of BrdU-labelled cells were negative for DCX and positive for the mature neuron marker NeuN (DCX^{-}; NeuN^{+}) (Fig. 3B,D). In *Nfix*^{iNestin} mice, however, there were one-third the number of DCX^{-}; NeuN^{+} cells (P=0.0009) (Fig. 3D), indicating that *Nfix*-deficient neuroblasts fail to generate dentate granule neurons efficiently.

The generation of adult-born dentate granule neurons is required for aspects of hippocampal-dependent learning in mice. For example, the suppression or enhancement of neurogenesis is typically associated with impaired (Deng et al., 2010) or enhanced (Sahay et al., 2011; Stone et al., 2011) encoding of new memories, respectively. We thus investigated whether the reduced production of dentate granule neurons in *Nfix*^{iNestin} mice resulted in impaired performance in an active place avoidance (APA) task. In this task, mice are placed in a rotating circular enclosure for 10 min, where, over the course of 5 days, they use external cues to learn to avoid a 60° segment of the arena that confers an electric shock upon entry. The ability of mice to learn to avoid the shock zone has previously been shown to rely on the generation of adult-born neurons (Vukovic et al., 2013). As expected, on the first day of testing, when the mice were naïve to the task, there was no difference between the number of shocks received by *Nfix*^{iNestin} or *Nfix*^{control} animals. However, by day 5 (P=0.031), control mice had improved their performance, such that they received significantly fewer shocks than on the first day of testing. In contrast, the number

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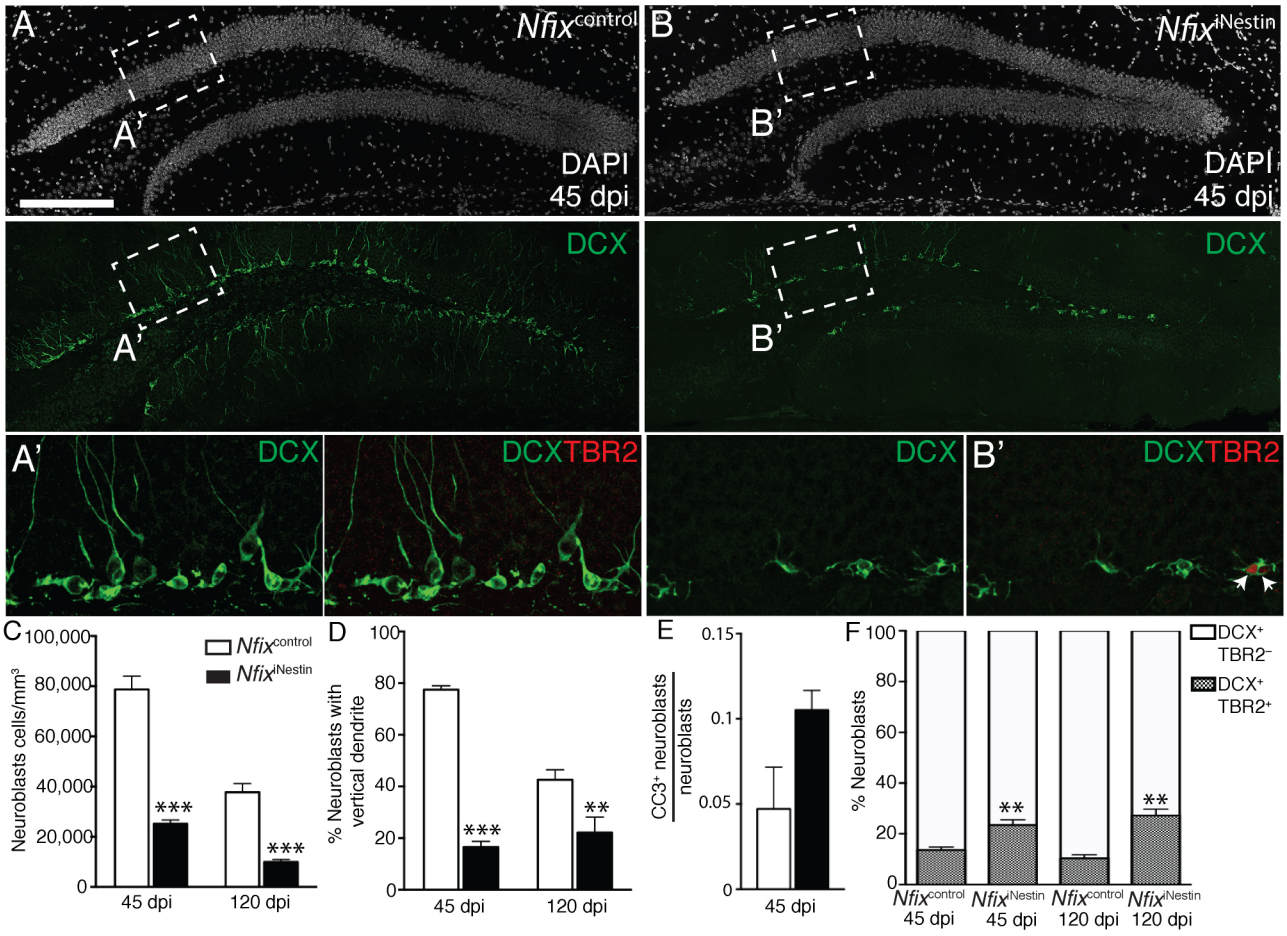

Fig. 2. Neuroblasts fail to mature in *Nfix*^{iNestin} mice. (A-F) The dentate gyrus of *Nfix*^{control} (A) and *Nfix*^{iNestin} (B) mice at 45 dpi stained for DAPI (white) and DCX (green). Boxed regions in A and B are shown at higher magnification in A′ and B′, respectively, and demonstrate DCX (green; left and right) and TBR2 (red; right) staining. At 45 and 120 dpi, *Nfix*^{iNestin} mice (B′) have fewer neuroblasts (DCX$^+$ cells) than do control mice (A,C). As a proportion, at both 45 and 120 dpi there were fewer neuroblasts in *Nfix*^{iNestin} mice that had a vertical dendritic process (D), whereas more co-expressed the apoptotic marker CC3 (E), although this was not statistically significant. Furthermore, significantly more neuroblasts in *Nfix*^{iNestin} mice expressed TBR2 in comparison with controls (F, arrows in B′). $^{**}P<0.01$, $^{***}P<0.001$. Graphs depict mean±s.e.m. from four mice per genotype at 45 and 120 dpi. Scale bar (in A): 250 µm (A,B); 50 µm (A′,B′).

of shocks received by *Nfix*^{iNestin} mice did not decrease during testing, and these animals performed significantly worse than controls on days 4 (P=0.0133) and 5 (P=0.0251) of testing (Fig. 3E-G). On all other parameters measured, such as distance travelled and speed of movement, *Nfix*^{iNestin} mice performed comparably to controls (Fig. S4). Furthermore, a primary SHIRPA screen (Harris et al., 2013) did not reveal any significant differences between control or mutant mice (Fig. S4). The findings demonstrate that the impaired differentiation of neuroblasts in *Nfix*^{iNestin} mice leads to a specific deficit in hippocampal-dependent learning and memory.

Nfix$^{iNestin\text{-}TD}$ mice generate fewer neurons in the adult hippocampus

We next used a lineage-tracing approach followed by histological and transcriptomic analyses to examine further the failure of neuroblasts to mature in *Nfix*^{iNestin} mice. We crossed *Nfix*^{iNestin} mice to a *flox-stop-flox* tdTomato reporter line, in which nestin$^+$ cells and all the progeny from these cells were permanently marked with red fluorescence following the administration of tamoxifen (Madisen et al., 2010). Here, the treatment group consisted of mice in which *Nfix* was deleted (*Nfix*$^{iNestin\text{-}TD}$) from nestin$^+$ cells (AH-NSCs and IPs), whereas in the control mice (*Wt*$^{iNestin\text{-}TD}$) labelled cells retained NFIX expression. We induced *Nfix* ablation and reporter gene expression by injecting tamoxifen, and performed our histological analyses at 60 dpi, analysing tdTomato expression in parallel with the mature neuron marker NeuN, and the neural progenitor marker SOX2. We made three important observations. First, there was a significant reduction in the total number of tdTomato$^+$ cells in the dentate gyrus of *Nfix*$^{iNestin\text{-}TD}$ mice compared with controls (P=0.0099) (Fig. 4A-C). Second, the reduction in total tdTomato$^+$ cells was correlated with a significant reduction in the total numbers of tdTomato$^+$-dentate granule neurons (P=0.007) (Fig. 4F). A similar finding was made when tdTomato$^+$-dentate granule neurons were evaluated as a relative proportion of the tdTomato$^+$ pool (P=0.0023) (Fig. 4I). Finally, although there was no effect on the total number of tdTomato$^+$; SOX2$^+$ progenitor cells in *Nfix*$^{iNestin\text{-}TD}$ mice (P=0.47) (Fig. 4D), as a proportion these cells were over-represented because of the significantly smaller tdTomato$^+$ population in *Nfix*$^{iNestin\text{-}TD}$ mice (P=0.018) (Fig. 4G). No effect was seen on the total number or proportion of tdTomato cells that were DCX$^+$ (Fig. 4E,H). Collectively, these findings are consistent with our previous data implicating NFIX as a central factor promoting neuronal differentiation in the adult mouse hippocampus.

We next isolated tdTomato$^+$ cells from treatment and control animals using fluorescence-activated cell sorting (FACS), and performed RNA-seq. As we had shown that NFIX is essential for

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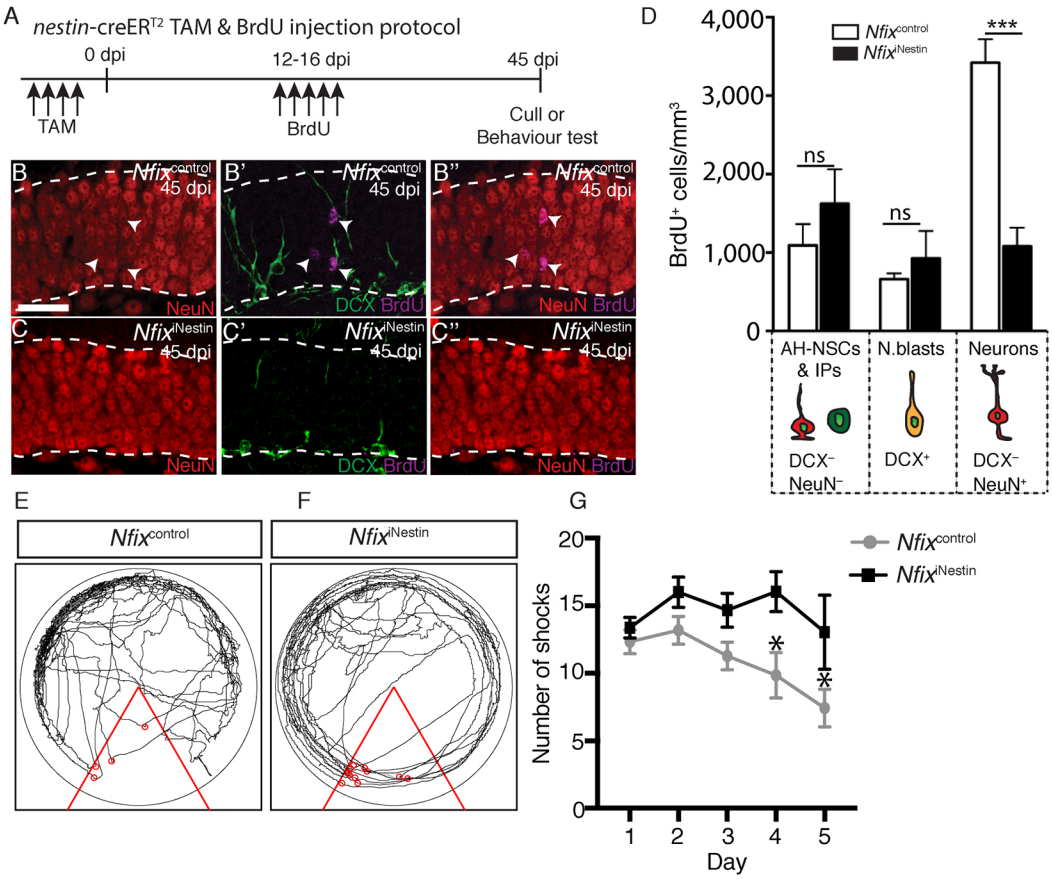

Fig. 3. *Nfix*^{iNestin} mice generate fewer mature granule neurons and have reduced performance in an APA task. (A) The tamoxifen (TAM)-BrdU injection regime used for *Nfix*^{iNestin} and *Nfix*^{control} mice. (B-C″) The dentate gyrus of *Nfix*^{control} (B-B″) and *Nfix*^{iNestin} (C-C″) mice at 45 dpi showing DCX (green), NeuN (red) or BrdU (magenta) staining, with dashed lines outlining the granule cell layer of the dentate gyrus. BrdU$^+$ dentate granule neurons, defined as NeuN$^+$; DCX$^-$ cells, are indicated by the arrowheads in B-B″. (D) There were significantly fewer BrdU$^+$ dentate granule neurons generated in *Nfix*^{iNestin} mice relative to controls. (E,F) Representative movement traces of *Nfix*^{control} (E) and *Nfix*^{iNestin} (F) mice during a 10 min trial of the APA task on the final day of testing (day 5). Red circles indicate administration of a shock within the designated shock area (marked by red lines). (G) During APA testing, control mice learnt the task so that they received significantly fewer shocks on day 5 compared with day 1. In contrast, the performance of *Nfix*^{iNestin} on day 5 of the protocol was not significantly different from that on day 1. Furthermore, *Nfix*^{iNestin} mice received significantly more shocks on day 4 and day 5 of the task than the control mice. **P*<0.05, ****P*<0.001; ns, not significant. Graph in D depicts mean±s.e.m. from four mice per genotype, graph in G depicts mean±s.e.m. from 21 mice per genotype. Scale bar: 30 µm.

the maturation of neuroblasts into dentate granule neurons, but that earlier precursors such as AH-NSCs and IPs were not significantly affected, we posited that the expression of mature neuronal markers would be reduced, and that, conversely, an over-representation of stem cell and early neuronal differentiation genes would be observed. In total, we identified 153 differentially expressed genes (Fig. 4J-L). Consistent with our hypothesis, many of the upregulated genes in the *Nfix*-deficient tdTomato$^+$ cellular cohort were members of the Notch pathway (*Hes5*, *Hes6*) or other progenitor cell markers and regulators (*Neurod2*, *Sox9*). Likewise, there were many cell adhesion molecules that were upregulated (*Dscam*, *Fezf2*, *Nrp1*, *Ptprz1*), genes typically associated with neuron recognition or neuron projection development. Interestingly, many genes associated with the inflammatory response were also upregulated in *Nfix*$^{iNestin\text{-}TD}$ animals, consistent with recent data suggesting that many of these molecules are highly expressed in nestin$^+$ hippocampal progenitors (Walker et al., 2016). Crucially, the mature neuronal marker *Camk1*, and *Prox1*, a marker of dentate granule neurons (Karalay et al., 2011), were downregulated. These histological and transcriptomic data further demonstrate that *Nfix* deletion from nestin$^+$ progenitors inhibits neuroblast differentiation and neuronal generation within the dentate gyrus of the adult mouse hippocampus.

Deletion of *Nfix* from hippocampal progenitors leads to aberrant oligodendrocyte production

Curiously, in the course of conducting the lineage-tracing experiments, we occasionally detected tdTomato$^+$ cells located on the hilar aspect of the SGZ within *Nfix*$^{iNestin\text{-}TD}$ mice. NFIX, as well as NFIA, and NFIB are pivotal regulators of astrocyte (Barry et al., 2008; Kang et al., 2012; Heng et al., 2014) and oligodendrocyte differentiation (Wong et al., 2007; Zhou et al., 2015; Rolando et al., 2016). For example, NFIX, NFIA and NFIB promote astrocyte development during the embryonic/postnatal development of the cerebral cortex, cerebellum and spinal cord (Barry et al., 2008; Kang et al., 2012; Heng et al., 2014). The relationship between NFI expression and the oligodendrocyte lineage is, however, more enigmatic. NFIX inhibits oligodendrocyte formation from postnatal SVZ progenitor cells *in vivo* and *in vitro* (Zhou et al., 2015). Conversely, de-repression of NFIB expression in AH-NSCs via deletion of *Drosha* promotes oligodendrocyte generation (Rolando et al., 2016). Under physiological conditions, AH-NSCs generate

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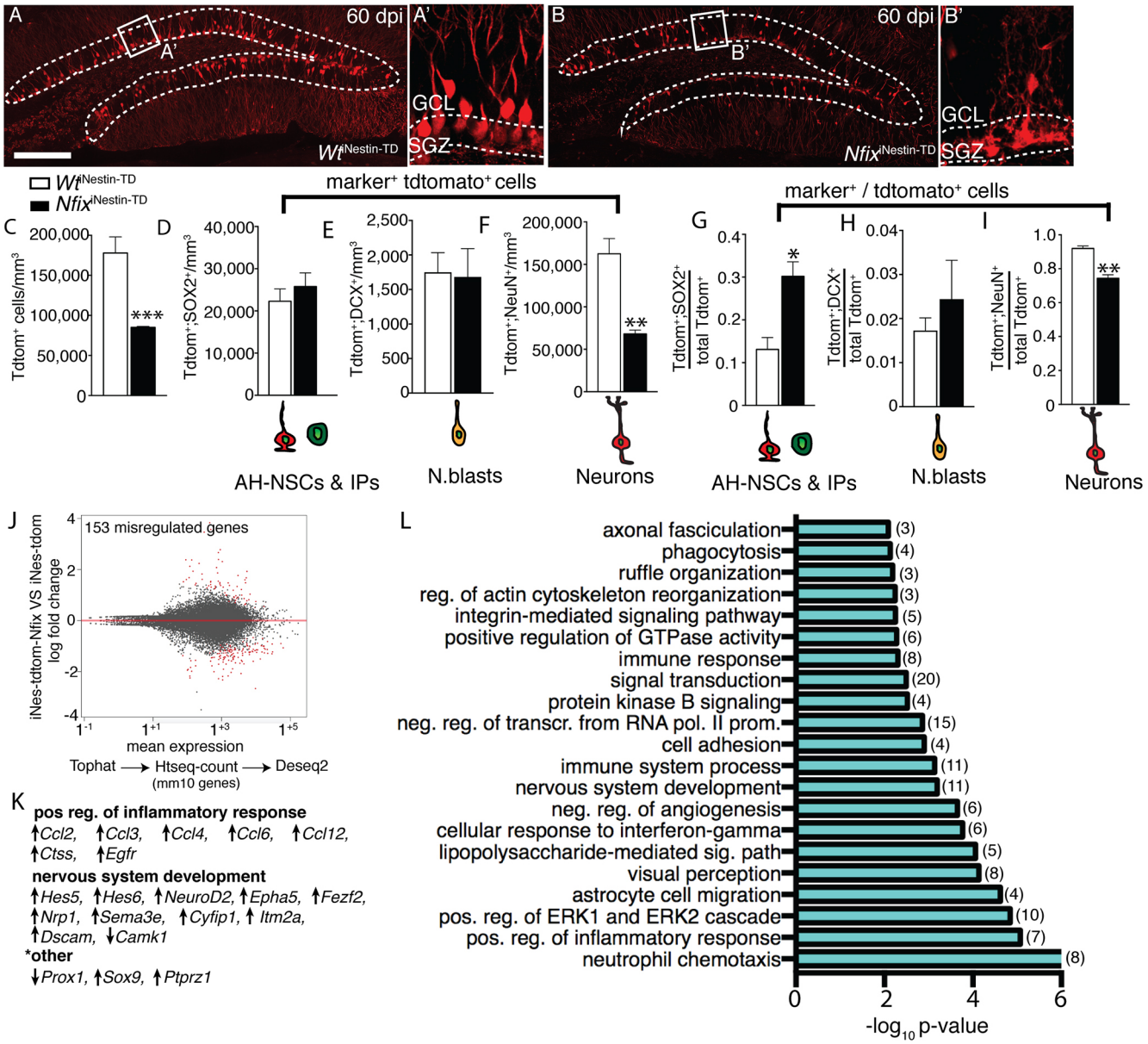

Fig. 4. *Nfix*$^{\text{iNestin-TD}}$ mice generate fewer neurons. (A-B′) *Wt*$^{\text{iNestin-TD}}$ (A) and *Nfix*$^{\text{iNestin-TD}}$ (B) mouse brains at 60 dpi stained for tdTomato (red), with the dashed lines outlining the dentate gyrus. The boxes in A and B are shown at higher magnification in A′ and B′, respectively, where the dashed lines demarcate the SGZ. (C) There were fewer tdTomato$^+$ cells in *Nfix*$^{\text{iNestin-TD}}$ mice relative to controls. (D-F) The number of tdTomato$^+$ cells that were SOX2$^+$, DCX$^+$ or NeuN$^+$ was quantified. There was no significant difference in the total number SOX2$^+$ (D) or DCX$^+$ (E) reporter cells between genotypes. However, there were significantly fewer NeuN$^+$ reporter cells in *Nfix*$^{\text{iNestin-TD}}$ mice compared with the controls (F). (G,H) As a proportion of the tdTomato$^+$ population, SOX2$^+$ cells were over-represented (G), whereas there was no change in DCX$^+$ cells (H). (I) As a proportion of the tdTomato$^+$ population, NeuN$^+$ cells were significantly under-represented relative to control mice. (J) MA plot of RNA-seq data of tdTomato$^+$ cells from *Wt*$^{\text{iNestin-TD}}$ and *Nfix*$^{\text{iNestin-TD}}$ mice at 45 dpi. (K) Genes and GO terms associated with early progenitor development were upregulated, whereas the late neuronal genes *Prox1* and *Camk1* were downregulated in the mutant. (L) GO terms ranked according to $-\log_{10}$ *P*-value. **P*<0.05, ***P*<0.01, ****P*<0.001. Graphs depict mean±s.e.m. from three mice per genotype, RNA-seq data generated from three mice per genotype. Scale bar (in A): 200 µm (A,B); 15 µm (A′,B′).

predominantly neurons, as well as a small number of astrocytes (Bonaguidi et al., 2011). However, AH-NSCs also have a latent tri-potency to generate all three differentiated neural cell types (Braun et al., 2015; Sun et al., 2015), as after forced *in vivo* expression of transcription factors such as *Olig2* (Braun et al., 2015), or after the deletion of neurofibromin 1 or *Drosha*, AH-NSCs are capable of generating oligodendrocytes (Sun et al., 2015). Although we did not detect a gene expression signature within our RNA-seq experiment that would indicate a shift towards astrocyte or oligodendrocyte production in *Nfix*$^{\text{iNestin-TD}}$ mice, this approach might not be sensitive enough to account for the paucity of these oligodendrocytic cells relative to the proportion of tdTomato$^+$ population. To investigate the production of astrocytes and oligodendrocytes, we co-stained for the astrocyte marker S100β or the pan-oligo marker OLIG2. We found no difference in the total number of tdTomato$^+$; S100β$^+$ cells between *Nfix*$^{\text{iNestin-TD}}$ and *Wt*$^{\text{iNestin-TD}}$ mice (*P*=0.13) (Fig. 5A-C). Surprisingly, however, we found a substantial increase in the number of tdTomato$^+$; OLIG2$^+$ cells in *Nfix*$^{\text{iNestin-TD}}$ compared with control *Wt*$^{\text{iNestin-TD}}$ mice, which were largely devoid of these cells in their hippocampi

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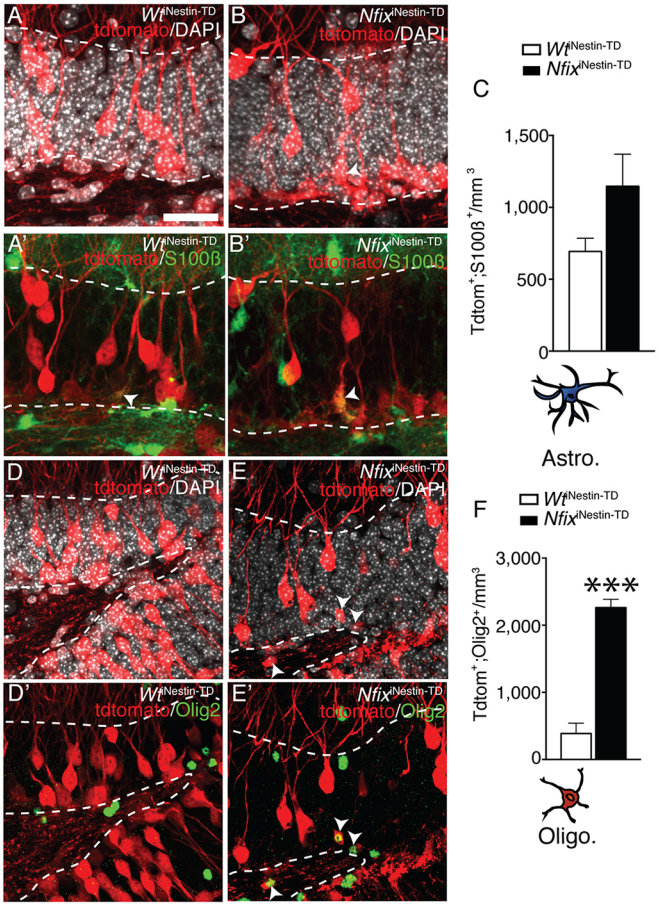

Fig. 5. *Nfix*$^{\text{iNestin-TD}}$ mice generate Olig2^{+} cells. (A-B′) *Wt*$^{\text{iNestin-TD}}$ (A,A′) and *Nfix*$^{\text{iNestin-TD}}$ (B,B′) mouse brains at 60 dpi were stained for tdTomato (red), S100β (green) and DAPI (white), with the dashed lines outlining the granule cell layer of the dentate gyrus. The arrowheads in A′ and B′ point to tdTomato^{+}; S100β^{+} astrocytes. (C) There was no significant difference in the number of tdTomato^{+} astrocytes generated between *Wt*$^{\text{iNestin-TD}}$ and *Nfix*$^{\text{iNestin-TD}}$ mice. (D-E′) *Wt*$^{\text{iNestin-TD}}$ (D,D′) and *Nfix*$^{\text{iNestin-TD}}$ (E,E′) mice at 60 dpi stained for tdTomato (red), OLIG2 (green) and DAPI (white). The arrowheads in E′ point to tdTomato^{+}; OLIG2^{+} cells of the oligodendrocyte lineage. (F) There were significantly more tdTomato^{+} cells of the oligodendrocyte lineage labelled in *Nfix*$^{\text{iNestin-TD}}$ mice than in controls. ***P<0.001. Graphs depict mean±s.e.m. from three mice per genotype. Scale bar: 40 µm.

(P=0.0099) (Fig. 5D-F). Therefore, deletion of *Nfix* from AH-NSCs leads to the aberrant production of a small number of cells expressing OLIG2, in addition to the more substantial defects seen in neuroblast maturation and survival. Although deletion of *Nfix* alone does not lead to a large-scale fate bias towards oligodendrocyte development, as seen with deletion of neurofibromatosis 1 (Sun et al., 2015), these results suggest that NFIX forms part of the genetic programme that represses the tri-potentiality of AH-NSCs.

NFIX expression is autonomously required for neuroblast maturation and survival

Is NFIX expression required cell-autonomously for neuroblast maturation and survival, or are the neuroblast maturation defects observed in *Nfix*$^{\text{iNestin}}$ mice due to the altered developmental trajectory of AH-NSCs following *Nfix*-deletion? To address this issue, we crossed our conditional *Nfix* mice to a line expressing a tamoxifen-inducible *cre*-recombinase under the control of the *Dcx* promoter (*Nfix*$^{\text{iDcx}}$) (Cheng et al., 2011). This line showed high recombination specificity in neuroblasts, and little to no recombination in AH-NSCs (Fig. S5). Because tamoxifen injections administered to these mice deletes *Nfix* from IPs and neuroblasts, but not from the AH-NSCs that generate these cells, tamoxifen injections were required weekly in order to continually deplete *Nfix* from these cells (Fig. 6A). Seven days after the final tamoxifen injection, we analysed the number of neuroblasts in *Nfix*$^{\text{iDcx}}$ and *Nfix*$^{\text{control}}$ mice, and found a significant reduction in the number of neuroblasts in the mutant strain (P=0.028) (Fig. 6B-D). Similar to *Nfix*$^{\text{iNestin}}$ mice, fewer of the remaining neuroblasts possessed a vertical dendritic branch (P=0.002) and more co-expressed TBR2 (P=0.047), findings consistent with impaired differentiation (Fig. 6E,F). Furthermore, BrdU labelling, followed by a 4-week chase, revealed that *Nfix*$^{\text{iDcx}}$ mice generated significantly fewer mature dentate granule neurons compared with control mice (P=0.035), and that more neuroblasts co-expressed cleaved-caspase-3 (P=0.015) (Fig. 6G-I). Finally, we also assessed the morphology of the few mature NFIX-negative neuroblasts that had, at least temporarily, escaped cell death. To do this, we crossed *Nfix*$^{\text{iDcx}}$ to the tdTomato reporter line to generate *Nfix*$^{\text{iDcx-TD}}$ mice and control *Wt*$^{\text{iDcx-TD}}$ mice. Seven days after the final tamoxifen injection, mature neuroblasts in *Nfix*$^{\text{iDcx-TD}}$ (defined as those cells having a primary neurite) had a substantially reduced dendritic complexity compared with controls (Fig. 6J-N). Therefore, the neuroblast maturation phenotype evident in *Nfix*$^{\text{iNestin}}$ animals was phenocopied following deletion of *Nfix* directly from DCX^{+} cells. These data are consistent with the hypothesis that *Nfix* expression is autonomously required by neuroblasts for the extension of a primary dendritic process and subsequent process branching, as well as the survival, maturation and integration of these cells into the hippocampal circuitry.

We next analysed the short-term cellular and transcriptional changes that occur upon deletion of *Nfix* from *Dcx*-expressing cells to determine the causative factors underlying the loss of this population. Seven days after the final tamoxifen injection to *Nfix*$^{\text{iDcx-TD}}$ and *Wt*$^{\text{iDcx-TD}}$, we analysed these reporter mice by immunohistochemistry, and by FACS followed by RNA-seq. In *Nfix*$^{\text{iDcx-TD}}$ mice, there were significantly fewer tdTomato^{+} cells in *Nfix*$^{\text{iDcx-TD}}$ compared with controls at 7 dpi (P=0.0163) (Fig. 7A-C). Given this finding, we posited that the transcriptomic analysis of tdTomato^{+} wild-type and mutant cells would reveal misregulation of key genes involved in neuronal maturation. We isolated tdTomato^{+} cells at 7 dpi using FACS, and performed RNA-seq on these samples. Consistent with our hypothesis, enriched gene ontology (GO) terms included neuron projection development, glutamate secretion, long-term synaptic potentiation and neuronal apoptosis (Fig. 7D-F). Of these, the enrichment of neuron projection development and neuronal apoptosis correlate strongly with the histological evidence of impaired dendrite formation and increased cell death of neuroblasts upon *Nfix* deletion. From this, we infer that NFIX expression is autonomously required by adult hippocampal neuroblasts to execute a programme of gene expression integral to dendrite formation and neuronal maturation.

The majority of neuroblasts in wild-type mice do not express protein markers of the oligodendrocyte lineage. However, a recent single-cell RNA-seq study of adult hippocampal neuroblasts revealed that a proportion of neuroblasts express putative oligodendrocyte-specific mRNAs, as well mRNAs encoding neuronal markers (Gao et al., 2016). In light of this, analysis of our gene expression dataset revealed that the most enriched GO term in our comparison of *Nfix*$^{\text{iDcx-TD}}$ and control mice, was, remarkably, ‘oligodendrocyte differentiation’. Misregulated genes under this

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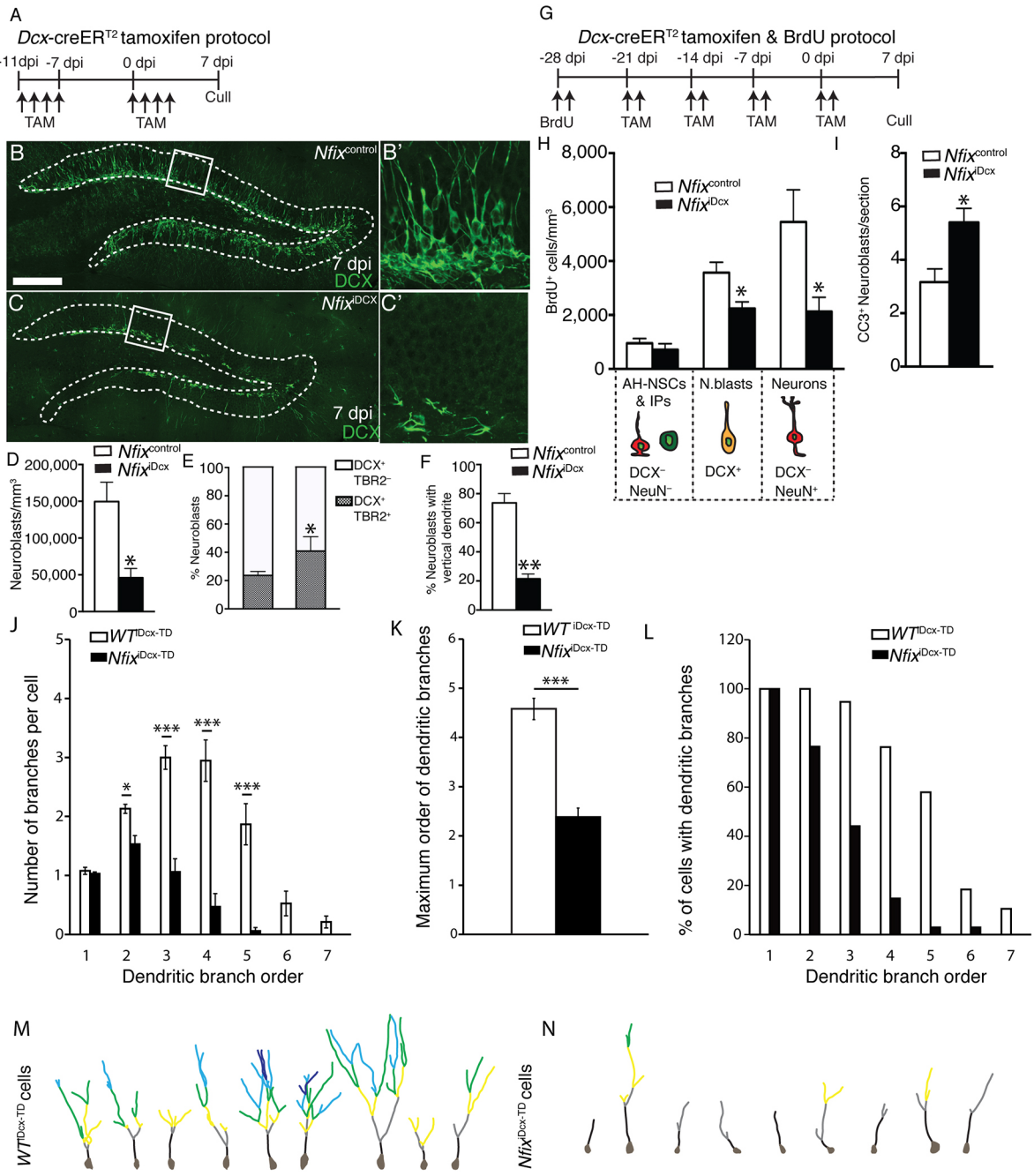

Fig. 6. Neuroblast-specific deletion of *Nfix* phenocopies deletion from AH-NSCs. (A) Tamoxifen (TAM) injection scheme for adult *Nfix*^{iDcx} and *Nfix*^{control} mice. (B-C′) *Nfix*^{control} (B) and *Nfix*^{iDcx} (C) mouse brains at 7 dpi stained for DCX (green), with dashed lines outlining the granule cell layer of the dentate gyrus. Boxed regions in B and C are shown at higher magnification in B′ and C′, respectively. (D-F) Compared with controls there were significantly fewer DCX^{+} cells in *Nfix*^{iDcx} mice (D). As a proportion, more neuroblasts in *Nfix*^{iDcx} mice co-expressed TBR2 (E), and fewer had a vertical dendritic process (F). (G) BrdU-TAM injection scheme for *Nfix*^{iDcx} and *Nfix*^{control} mice. (H) 4 weeks after BrdU administration there were significantly fewer BrdU^{+} neuroblasts (DCX^{+}; NeuN^{-}) and BrdU^{+} mature neurons (DCX^{+}; NeuN^{+}) in *Nfix*^{iDcx} mice compared with the controls. Additionally, more neuroblasts in *Nfix*^{iDcx} mice co-expressed CC3 at 7 dpi in comparison with the controls (I). (J-L) Using the experimental protocol outlined in A, the dendritic features of *Nfix*$^{iDcx\text{-}TD}$ and *Wt*$^{iDcx\text{-}TD}$ neuroblasts were examined. Surviving *Nfix*$^{iDcx\text{-}TD}$ neuroblasts had fewer branches per cell (J) and reduced maximum order of dendritic branches (K,L). (M,N) Representative tracings of control (M) and *Nfix*$^{iDcx\text{-}TD}$ (N) cells. **P*<0.05, ***P*<0.01, ****P*<0.001. Graphs in D-F depict mean±s.e.m. from three mice per genotype, graphs in H,I depict mean±s.e.m. from four mice per genotype, graphs in J,K depict mean±s.e.m. of 34 control and 38 *Nfix*$^{iDcx\text{-}TD}$ neuroblasts. Scale bar: 250 µm (B,C); 50 µm (B′,C′).

grouping included oligodendrocyte precursor/differentiation markers such as *Ntrk2* and *Ptprz1*, which were ∼3-fold upregulated in *Nfix*$^{iDcx\text{-}TD}$ mice compared with controls, as were the pan-oligo markers *Olig1* and *Olig2*. The expression of the mature oligodendrocyte markers *Cnp*, *Mbp* and *Mog* were unaffected, suggesting that *Nfix* deletion from neuroblasts leads to a specific upregulation of genes associated with early oligodendrocyte development (Fig. 7G).

To rule out the possibility that the increased oligodendrocyte precursor-specific gene expression signature in the *Nfix*$^{iDcx\text{-}TD}$ mice was due to non-specificity of the *cre*-recombinase, i.e. oligodendrocyte precursor cells potentially being labelled and

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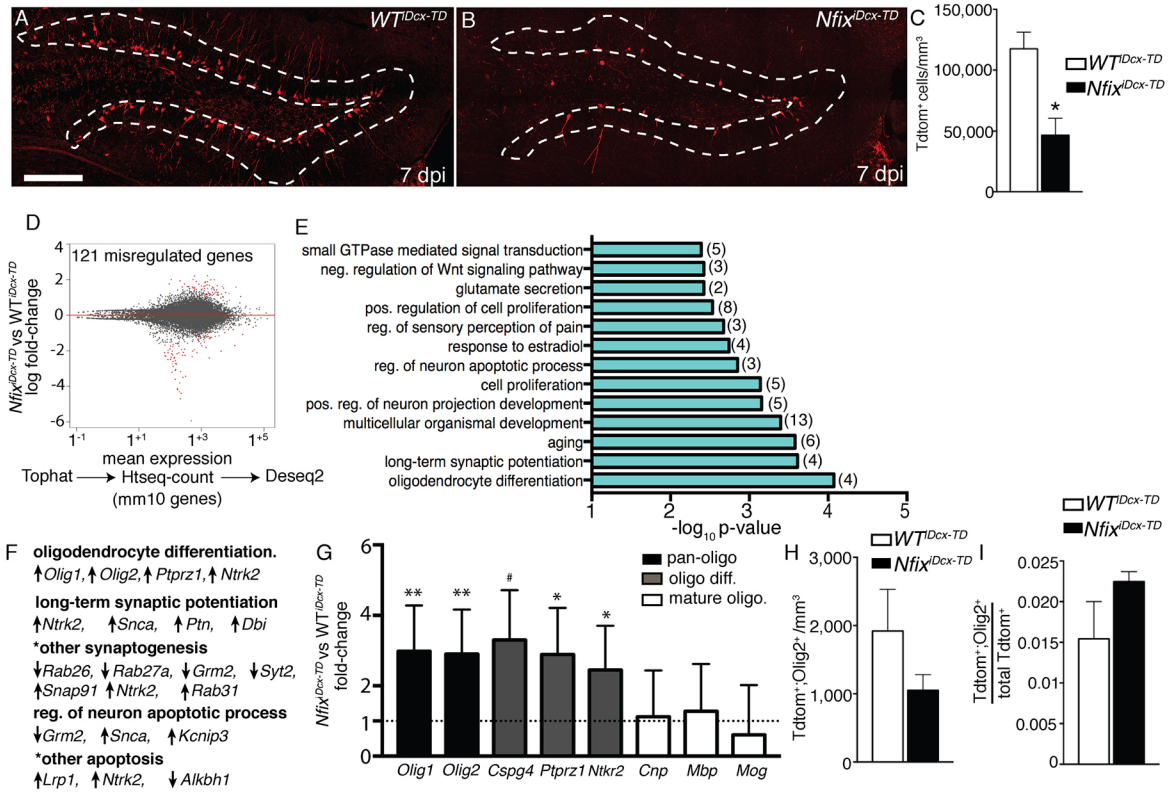

Fig. 7. Neuroblast-specific deletion of *Nfix* leads to expression changes in neuronal maturation and in oligodendrocyte precursor genes. (A,B) *Wt*$^{iDcx\text{-}TD}$ (A) and *Nfix*$^{iDcx\text{-}TD}$ (B) mouse brains at 7 dpi stained for tdTomato (red), with the dashed lines outlining the granule cell layer of the dentate gyrus. (C) There were significantly fewer tdTomato$^+$ cells in *Nfix*$^{iDcx\text{-}TD}$ mice relative to the controls. (D) MA plot of RNA-seq data of tdTomato$^+$ cells from *Wt*$^{iDcx\text{-}TD}$ and *Nfix*$^{iDcx\text{-}TD}$ mice at 7 dpi. (E) GO terms ranked according to $-\log_{10}$ *P*-value. GO terms associated with neuronal maturation and oligodendrocyte differentiation were enriched. (F) Curated list of genes associated with enriched GO terms. (G) Fold change of oligodendrocyte lineage genes, categorised according to whether they are expressed throughout the entire lineage (pan-oligo), during differentiation (oligo diff.) or by mature oligodendrocytes (mature oligo.). FDR-adjusted *P*-value: $^{\#}P<0.1$; $^{*}P<0.05$, $^{**}P<0.01$. (H,I) No difference in the total number of OLIG2$^+$; tdTomato$^+$ cells (H) or the proportion of these cells in reference to the total tdTomato$^+$ pool (I) was observed. Graphs in F depict mean±s.e.m. of RNA-seq data generated from three mice per genotype. Graphs in H and I depict mean±s.e.m. of three *Nfix*$^{iDcx\text{-}TD}$ and four *Wt*$^{iDcx\text{-}TD}$ mice. Scale bar: 200 µm.

amplified upon *Nfix* deletion, we examined the identity of tdTomato$^+$ cells in *Wt*$^{iDcx\text{-}TD}$ at 7 dpi. The vast majority of tdTomato$^+$ cells either expressed DCX or were DCX$^-$ with a neuronal morphology indicating they were dentate granule neurons that had recently lost DCX expression. In contrast, very few tdTomato$^+$ cells expressed OLIG2 (48/3113 cells, 1.54%), demonstrating the specificity of the *cre*-recombinase. We next examined whether the upregulation of oligodendrocyte mRNA in *Nfix*$^{iDcx\text{-}TD}$ mice was due to the presence of greater numbers of tdTomato$^+$; OLIG2$^+$ cells in these mice. There was no significant difference in the total number of tdTomato$^+$; OLIG2$^+$ cells in *Nfix*$^{iDcx\text{-}TD}$ compared with controls (P=0.297) (Fig. 7H). Consistent with this, tdTomato$^+$; OLIG2$^+$ cells did not account for a more significant proportion of the reporter-positive pool in *Nfix*$^{iDcx\text{-}TD}$ mice than in controls (P=0.257) (Fig. 7I). We also found no difference in the number of tdTomato$^+$ astrocytes between genotypes (data not shown). Therefore, *Nfix* deletion from neuroblasts leads to a de-repression of oligodendrocyte gene expression but not an increase in oligodendrocyte cell number, probably because these cells either do not have the capacity to mature fully, or that this fate change is not detectable because of the high rates of neuroblast cell death.

Together, our results demonstrate that NFIX expression is absolutely required for the survival and timely generation of adult-born neurons within the mouse hippocampus. NFIX enacts this function by driving the programme of neuronal differentiation within neuroblasts. Remarkably, loss of *Nfix* leads to oligodendrocyte differentiation in a proportion of AH-NSCs and the elevated expression of oligodendrocyte mRNA within neuroblasts. These data demonstrate that NFIX functions as part of the gene regulatory network that suppresses the latent tri-potentiality of AH-NSCs, and, crucially, that NFIX inhibits the expression of genes central to oligodendrocyte fate within the lineage-restricted progeny of AH-NSCs within the adult hippocampus.

DISCUSSION

Studies in rodents have begun to reveal the key transcription factors required for the different stages of adult hippocampal neurogenesis. Transcription factors integral to regulating cell-cycle entry (such as FOXO and ASCL1), stem cell maintenance (such as PAX6 and REST) and the production of IPs (such as TBR2) have been identified as being central to neurogenesis within this niche of the adult brain (reviewed by Urban and Guillemot, 2014). The NFI family of transcription factors has been extensively described in the developing brain, and has been implicated in multiple neurodevelopmental disorders (Malan et al., 2010), but how these factors function in the adult hippocampus is unclear. A recent study revealed a role for NFIB in promoting oligodendrogenesis within the adult SGZ (Rolando et al., 2016). Here, we present a contrasting

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role for NFIX within the adult hippocampus, revealing that NFIX drives a programme of neuroblast differentiation, as well as suppressing the latent potentiality of hippocampal stem and progenitor cells to generate oligodendrocytes.

Although our present study identifies neurogenic functions for *Nfix* in the adult hippocampus, we reveal two key differences with regards to its function within the embryonic brain. First, the relative stages of neuronal lineage progression regulated by NFIX are different for embryonic stem cells and precursor cells within the adult hippocampus. Second, *Nfix* deletion has differential effects on neuronal survival in cells of the embryonic brain versus the adult hippocampus. In the developing dorsal forebrain, NFIX promotes the asymmetric division of radial glial stem cells and the subsequent production of IPs (Harris et al., 2016), which is the earliest cell fate choice that occurs during neuronal differentiation. As a result, radial glial stem cells undergo more self-expanding (proliferative) divisions in *Nfix*$^{-/-}$ mice, thereby extending the neurogenic period. This results in the production of more neurons and postnatal macrocephaly in this mouse line, without any reported effects on neuronal survival (Campbell et al., 2008; Heng et al., 2014; Harris et al., 2016). In contrast, here we reveal that the deletion of *Nfix* in AH-NSCs does not markedly affect the production of IPs (Fig. 1). The expression of NFIX within the SGZ neurogenic niche provides insights into these differences, as NFIX expression is highest within DCX^{+}; TBR2^{-} neuroblasts. The failure of *Nfix*-deficient neuroblasts to mature culminates in the death of many of these cells, which is reflected in the hippocampal-dependent behavioural deficits evident in the *nestin-creER*^{T2} knockout line (Fig. 3). Therefore, although NFIX functions to promote neuronal differentiation both during development and in adult hippocampal precursor cells, the consequences of NFIX deletion on neuronal survival and neuronal number vary between the two contexts. Whether these differences in NFIX function reflect interactions with alternative co-factors, or disparities in the intrinsic chromatin architecture of neural progenitors within these varying contexts, remains to be investigated.

NFIX has previously been hypothesised to play a central role in maintaining AH-NSC quiescence. Using an *in vitro* model of NSC quiescence, Martynoga and colleagues (2013) demonstrated that NFIX was a major factor that bound to quiescence-specific enhancer regions, and that loss of *Nfix* led to increased neural stem cell proliferation within this culture system. Consistent with this finding for NFIX in mediating stem cell quiescence, the authors reported an increased number of proliferating AH-NSCs cells in the hippocampus of postnatal day 20 *Nfix*$^{-/-}$ mice. However, an alternative explanation for this phenotype lies in the finding that these mice display developmental deficits within the dorsal telencephalon (Martynoga et al., 2013; Heng et al., 2014). Given the neurogenic roles for NFIX *in vitro*, we posited that the conditional, inducible deletion of *Nfix* from AH-NSCs would lead to a substantial increase in AH-NSC proliferation and subsequent depletion of this population. Intriguingly, the findings of our study did not support this hypothesis. *Nfix* deletion from AH-NSCs led to a temporary increase in AH-NSCs at 14 dpi, but total AH-NSC cell number remained unchanged even as long as 1 year following *Nfix* deletion. There are a number of explanations that could account for the limited effect of *Nfix* deletion on AH-NSC quiescence/proliferation. First, although NFIX was found to bind the majority of quiescence-specific enhancers *in vitro*, these experiments utilised neural stem cells derived from embryonic stem cells that could have a substantially different epigenetic landscape than AH-NSCs *in vivo* (Martynoga et al., 2013). Second, the *in vitro* experimental protocol removes stem cells from their niche, which *in vivo* comprises a dense network of blood vessels, immune cells, neurotransmitters and chemical signals (reviewed by Gonçalves et al., 2016). The complexity of the niche signals might have buffered against any subtle effects of *Nfix* deletion on the relative quiescence of AH-NSCs, thereby leading to only a transient effect. Finally, given the functional overlap between NFIX and other NFIs proteins during brain development (Barry et al., 2008; Piper et al., 2010; Heng et al., 2014), it is feasible that other NFI family members could also regulate AH-NSC quiescence within the mature dentate gyrus. As such, another interesting avenue of future research would be to determine whether the compound deletion of *Nfib* or *Nfia*, in conjunction with *Nfix* deletion, would result in a more substantial and sustained loss of AH-NSC quiescence.

In this study, we found that NFIX suppresses the capacity of AH-NSCs to generate oligodendrocytes. AH-NSCs do not generate oligodendrocytes under basal conditions. For example, Bonaguidi and colleagues (2011) demonstrated that there were no reporter-positive cells within 300 clones (generated by a low-dose tamoxifen injection regime in *nestin-creER*^{T2} animals crossed to a reporter line) that co-labelled with oligodendrocyte markers. Similar findings of the low propensity for oligodendrocyte production by AH-NSCs have been made using a *glast-creER*^{T2} line (Sun et al., 2015). Although a caveat to these approaches pertains to the inefficiencies of these *cre* drivers to label the full diversity of hippocampal precursors (Braun et al., 2015), this is clearly a restricted process.

In contrast, a number of recent studies have highlighted that, at least under certain circumstances, AH-NSCs can generate oligodendrocytes, and thus the pathways that act to suppress the production of oligodendrocytes from AH-NSCs *in vivo* now emerge as a topic of intense investigation. For example, it was recently reported that AH-NSCs possess a substantial tri-lineage potential, as overexpression of *Olig2*, *Sox10* or *Ascl1* using a retrovirus was sufficient to drive a significant proportion of AH-NSCs to generate oligodendrocytes (Braun et al., 2015). A more dramatic effect was seen upon deletion of neurofibromin 1, whereupon large numbers of AH-NSCs generated oligodendrocytes (Sun et al., 2015). Our results suggest that NFIX also functions to suppress the tri-potentiality of AH-NSCs. However, unlike earlier studies, which solely used *cre*-recombinase lines and viruses that predominantly labelled AH-NSCs, we also detected de-repression of oligodendrocyte genes upon *Nfix* deletion from IPs and neuroblasts. Our data therefore suggest that targeting barriers of latent lineage potential, even within cells that are ostensibly considered to be committed to neuron production, could be an avenue to generate additional plasticity. It would interesting to examine whether the conditional deletion of neurofibromin 1 (Sun et al., 2015) using a *Dcx-creER*^{T2} driver might be sufficient to convert a substantial number of adult hippocampal neuroblasts to become oligodendrocytes without the concurrent cell death that occurs upon *Nfix* deletion. There is precedent for similar cell-type conversions between disparate cell types. For example, it was recently shown that adult striatal astrocytes exhibit a latent neurogenic programme that is actively suppressed by Notch signalling (Magnusson et al., 2014) and elicited by injury (Nato et al., 2015).

In conclusion, this study has uncovered the contrasting dual roles of NFIX during adult hippocampal neurogenesis. First, we found that NFIX drives a programme of neuroblast differentiation, and, second, that it suppresses the latent potency of hippocampal precursor cells to generate oligodendrocytes, thereby significantly

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enhancing our understanding of the transcriptional control of adult hippocampal neurogenesis and the latent lineage potential of AH-NSCs, as well as their IP and neuroblast progeny.

MATERIALS AND METHODS

Animal ethics

Experiments were approved by The University of Queensland Animal Ethics Committee (AEC approval numbers QBI/353/13/NHMRC and QBI/383/16), and were performed in alignment with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. For details of mouse strains and the regime for tamoxifen and BrdU injections, please refer to the supplementary Materials and Methods. All injections/experimental time points began when mice were between 8 and 10 weeks old. Both male and female mice were used throughout the study.

Antibodies, immunofluorescence and cell counts

Every sixth section, spaced 300 µm apart (six sections per brain) was mounted on slides for immunofluorescence staining. Exceptions to these were analyses of tdTomato mice for which three sections per brain were analysed. Sections were immunostained as previously described (Harris et al., 2016). For details of the staining protocol, antibodies, imaging and measurements, see supplementary Materials and Methods.

Cell sorting and RNA-seq

The dentate gyri of reporter mice were dissected in ice-cold PBS (Hagihara et al., 2009). RNA was amplified according to the Smart-seq2 protocol (Picelli et al., 2013) using 12 cycles for *Nfix*$^{\text{iNestin-TD}}$ animals and 13 cycles for *Nfix*$^{\text{iDcx-TD}}$ animals. For full protocol, see supplementary Materials and Methods.

Processing and analysis of RNA-seq data

Data were analysed on the public Galaxy server, Galaxy version 16.07 (Goecks et al., 2010). Raw reads were aligned to the mouse genome (mm10) using Tophat (Trapnell et al., 2009). Transcript levels were quantified in HTSeq-count (Anders et al., 2015) by mapping to known mouse (mm10) RefSeq (NCBI) protein-coding sequences, which were downloaded in a GTF format from the UCSC table browser. In HTSeq-count the overlap mode used was 'union', and the strandedness set to 'no'. Differential gene expression analysis was then performed using the DeSeq2 package (Love et al., 2014). GO analysis was performed in DAVID (Huang et al., 2009a,b). For DAVID analysis, the background gene set included any gene with a non-zero count in the DeSeq2 output (Timmons et al., 2015). To summarise redundant GO terms from DAVID, the program REVIGO and the semantic similarity measure Simrel were employed (Supek et al., 2011).

Behavioural analysis

The active place avoidance task was used to assess hippocampal-dependent spatial learning (for details, see supplementary Materials and Methods), and a primary SHIRPA screen was used to assess gross phenotypes (Harris et al., 2013).

Statistical analyses

The parameters of our statistical testing approach were specified prior to data collection. Two-tailed unpaired Student's *t*-tests were performed when comparing two groups. For experiments involving two independent variables, two-way ANOVA was performed, with repeated measures if applicable; *P*-values from two-way ANOVA are reported in the text. Any significant main effect of genotype detected by two-way ANOVA was followed by multiple *t*-tests using a pooled estimate of variance where appropriate.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.H., R.M.G., M.P.; Formal analysis: L.H., O.Z., O.C., J.F., E.M., S.O., T.J.H., T.H.J.B., J.I.H.; Resources: R.M.G.; Writing - original draft: L.H., M.P.; Writing - review & editing: L.H., O.Z., O.C., T.H.J.B., J.I.H., R.M.G., M.P.; Supervision: M.P.; Project administration: M.P.; Funding acquisition: M.P.

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Data availability

RNA-seq data have been deposited in Gene Expression Omnibus under accession number GSE109817.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.155689.supplemental

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